IN VITRO ANTITUMOR POTENTIAL OF SOYBEAN LECTIN, ISOLATED FROM *Glycine max*

Thesis submitted to Department of life science for the partial fulfillment of the M.Sc. Degree in Life science

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राष्ट्रीय प्रौद्योगिकी संस्थान, राउरकेला

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CERTIFICATE

This is to certify that the thesis entitled **"In Vitro Antitumor Potential of Soybean Lectin, Isolated from *Glycine max*"** which is being submitted by Mr. Sandeep Dey, Roll No. 411LS2046, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I do hereby declare that the Project Work entitled **"In vitro antitumor potential of soybean lectin, isolated from *Glycine max*"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Sujit Kumar Bhutia, Asst. Professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

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Place: Sandeep Dey

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Sandeep Dey

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LIST OF ABBREVIATIONS

PBS	Phosphate Buffer Saline
et al	And others
Rpm	Rotation Per minute.
Conc	Concentration
Hrs	Hours
L	litre
M:	Milli gram
pH	Hydrogen concentration
NaOH	Sodium hydroxide
Na2CO3	Sodium carbonate
APS	Ammonium per sulphate
TEMED	N,N,N',N'-tetramethylenediamine
KNaC4H4O6	Potassium sodium tartarate
SDS-PAGE	Sodium Dodecyl sulphate Polyacrylamide Gel electrophoresis.
BSA	Bovine serum albumin
KH2PO4	Potassium Dihydrogen Phosphate
K2HPO4	Potassium hydrogen phosphate
(NH4)2SO4	Ammonium Sulphate
Pvt .Ltd	Private limited
kDa	kilo dalto
Sbl	soybean lectin
°C	Degree Celsius
Min	Minute
H	Hour
M	Molar
mM	Mili molar
L	Liter
mL	Milli Liter
Kg	Killogram
gm	Gram
mg	Milligram
µg	Micro gram
µm	Micro meter
% :	Percentage
OD	Optical Density
EDTA	Ethylene diamine tetra acetate

ABSTRACT

Soybean lectin is one of the most important lectins which have anti-cancer activity, primarily confirmed by MTT and trypan blue exclusion assay in various cancer cell lines. The cell viability percentage increases in decreasing dose concentration of the soy lectin. Besides the above it also induces laddering of the DNA in a dose dependent manner. DNA fragmentation was occurred after treating the soya lectin in HeLa cell lines. Isolation of the soya lectin by using Sepharose 4B column is a novel approach. Characterization of this lectin was done by performing Haemagglutination assay and determining molecular weight by both Native and SDS-PAGE which was found to be 120 kD and 30 kD respectively.

Keywords: Soybean lectin, SBL, Affinity chromatography, SDS-PAGE, haemagglutination assay, MTT Assay, Trypan Blue Exclusion assay

INTRODUCTION

Traditional cancer can be treated by Peptide-based therapies which offer the potential for non-genotoxic, genotype-specific alternatives, or adjuvants (5). Natural peptides lay a key role in maintaining the physiological and different biochemical processes in the body by their direct or indirect involvement. These are the short chains of around 50 amino acid residues which are being stabilized by several factors and joined to each other by means of disulfide bonds. They have high biological importance as they regulate many of the biological activities which are very valuable for our body. These peptides have several important roles in cell signaling and other biochemical actions which delivers biochemical messages. These may play role as agonists, antagonists, modulators, mediators, hormones, effectors, cofactors, activators, stimulators, and so on. Peptides have many functional activities like they are potentially antigenic, can be used as pathological biomarkers, signaling molecules, antimicrobial compound, immunogenic molecule and enzymes modulators or inhibitors as they can be easily detected and quantified in biological fluids. Natural peptides can be derived from both plants and animals. Venom fluids from the venomous animals are the complex mixtures of several peptides which either post transcriptionally modified or phosphorylated .They bind with different target proteins ,receptors ,ion channels with higher affinity. Natural peptides can also act as therapeutic molecule or they may be component in designing the drug molecules. Traditionally mass spectrometry analysis and chromatography techniques are used for purification and characterization of these peptides. Still several new techniques are now in the market which helps in deciphering the mystery behind these peptides and their functions. Next gen sequencing, protein homology modeling etc. is helpful for the vivid study of the transcriptome (25).

Two phenomena are mainly responsible for by which the multi-cellularity and adulthood in an organisms, that is cell division and differentiation. Any abnormality, change or aberration in this system leads a clump of malfunctioning cells having some malignant property. The loss of regulation and autonomous cell multiplicity leads to the cancerous or neoplastic growth. Cancer occurs when the cell gone wild and divide unusually. It affects people throughout the world both in developed and developing countries. In United States mortality associated with lung and bronchus cancer among women continues to increase and lung cancer is expected to account for 25% of all female cancer deaths in the year2000 . treatment of cancer is still not possible if it has

spread over the body.so metastasis(migration of cancer cells to different via blood stream and lymph channels to different body parts) and proliferation of neoplastic cells are mainly responsible for determining the fate of a cancer patient. Cancer begins with a single cell but in later stage due to the mutation in cellular mechanism they perform an uncontrollable division of the cells which leads to malignancy causing cancer. Metastasis involves cell to cell interaction which has diverted the cancer research towards itself; scientists are more interested to know that what are the causes that the cancerous cells can easily move from one part to the other part of the body and what can be several preventive mechanisms to stop the process. Cell membrane and cellular has several glycoconjugates, glycoproteins, carbohydrate moieties etc. These can be detected by several protein biomarkers like lectins. Lectins are the glycoproteins which have the specificity towards several carbohydrate moieties present on the cell surface, ac t as differentiating markers to study cancers and metastatic cell lines. The binding of lectins to the specific carbohydrate help in detecting the carbohydrate residues present on the cell surface.

What are Lectins?

Lectins are proteins which have carbohydrate specificity and have the property to agglutinate erythrocyte. These are otherwise called as the glycoproteins having molecular weight of 60-100 kda (64). The word lectin has come from the Latin word '*legere'* that means "to bind" or "to pick and choose" by William Boyd in 1954.This was first isolated by still mark in 1888 and are mainly present in grains and legumes in high amount. These have one non catalytic domain that reversibly binds with the specific monosaccharaides and oligosaccharides. They can be classified according to their specificity to carbohydrate and also according to their overall structure in to merolectins, hololectins, chimerolectins and superlectins or be grouped into different families like mannose-binding lectins, legume lectins, ribosome-inactivating proteins.

Lectins act as the primitive protective system in the plant those are analogous to an antibody but in a non-immune model. Not only in plants have these also helped as the immune protectant in human body. There are various kinds of lectins with various different structures and but the only thing that they are resembling to each other is there is ability to bind towards a sugar molecule.it binds with the terminal sugar molecule and forming a glycoconjugates on the cell membrane.

Molecular Structure of Lectins

lectins have the basic property of binding towards specific saccharides.it is having two sub units combined to five different forms of non-covalently bound tetramers.as it contains endogenous saccharide receptors so it has specificity towards saccharides.

Metal Binding Sites

It is mainly helpful to maintain the native structure of the leguminous lectins.

Hydrophobic Sites

Stability in the protein structure is due to the hydrophobic interactions.

Glycosylation Sites

Maximum of the lectins are thought to be glycoproteins except concanavalin A, lentils lectin, and wheat germ agglutinin which have no covalently attached carbohydrates. Asparagine- X-threonine/serine is characteristic of glycosylation present in peptide sequence of all glycoprotein lectins containing.

Carbohydrate Binding Sites

Lectins differ markedly in their sugar-binding specificity. An identical or\homologous B- turn structures are found when secondary structure of several lectins is compared (64).

FUNCTION OF LECTINS

As anti-cancerous agent:-

Membrane glycosylation helps in cell adhesion, migration and communication. But in malignant cells these properties are totally lost due to the altered cell surface carbohydrate expression. Lectins are mainly used as the therapeutic and diagnostic tool for the treatment of cancer. They are helpful for the investigation of the changes occurred in the structural and functional activity of the carbohydrate complexes during pathological and physiological process which helps for the detection of the cancer. Due to the ability of the lectins to detect the carbohydrate moieties and

the cytotoxicity against them has made lectins a superior field of research in the field of cancer Biology.

Lectin Inducing Programmed Cell Death

Type I programmed cell death or Apoptosis is a complex but highly defined cellular program of cell death (Cotter, 2009). Autophagy refers to an evolutionarily conserved, multi-step lysosomal degradation process in which a cell degrades long-lived proteins and damaged organelles. (62).several plant lectins can induce apoptosis or may have anti proliferative property towards cancer cells (15).but there are some lectins which result in inducing autophagy after binding to the cell surface receptors on the cancer cells .

It has been shown by many research that Dark red kidney bean haemagglutinin (PHA-E) can employ an anti-proliferative activity toward leukemia L1210 cells spread of breast cancer MCF-7 cells and hepatoma HepG2 cells can be obstructed by small glossy black soybean lectin . Recent studies have shown that retardation of proliferation of L1210 cells and HepG2 cells can be done by that Del Monte banana lectin. Autumn purple bean lectin can induce production of apoptotic bodies and French bean haemagglutinin has been shown to induce breast cancer MCF-7 cell apoptosis.

The current project focuses upon the isolation and purification of protein where a novel method has been adopted for isolation. After isolation the efficacy of protein was checked by testing the agglutination of erythrocyte for the presence of protein of interest. Then the protein was used to check the effect of it on the cancerous cell lines.

REVIEW OF LITERATURE

Soybean (Glycine max), one of the member of legume family are native to East Asia. It is an ancient legume used up worldwide most commonly in Asian countries, such as China, Japan, Korea, Taiwan and Indonesia . 20 to 80 g of average traditional soy foods are consumed on a daily basis by the Populations from these countries including soybean, soybean sprouts, toasted soy protein flours, soy milk, tofu, and fermented soy products, such as tempeh, miso, natto, soybean paste and soy sauce (62). Whereas 1 to 3 g of daily consumption is done by Western populations by means of different soy products (18).

These are introduced to western world since the 20^{th} century. These can be grown on various soil and climate also. Soybeans are mature on the pod, ripen into hard, dry beans and they mostly retain the yellow colour after maturation.one fifth of all plant proteins, consumed by people are provided by the seed legumes Seed legumes on a global basis (32;49). These are the valuable sources of protein and oil for both human and animals (33). Otherwise known as the Glycine max having many bioactive properties (33;2;50). Soybeans can be prepared by several way for human and animal consumption. Soymilk is produced from the seeds which are very nutritious and proteinaceous in nature. They have been categorized under fabaceae family and considered as the cheapest and richest source of protein. These can also be cooked and then fermented using vinegar and the fungus *Rhizopus oligosporus* to make tempeh. The waste soy flour create textured vegetable protein (TVP), mainly used as the meat substitutes. Soy seeds have 17 % oil and 63 % meal, 50 % of which is protein. It has no starch (17). Generally production of soybean is referred to as216, 144, 262 tones and harvested area of 94,899,216 hectors.

In old days soy was valued as a valuable green manure for the nitrogen fixing ability of its rots.it was not been eaten before due to its indigestibility and gas forming activity and also of its anti-nutritive effect of its many constitutes. Soy has the chemicals which can mimic estrogen and lower testosterone levels.it had assisted the sexual abstinence of monks, so taken as the regular food of them. They call it as “meat without a bone”I.t has low level of methionine essential sulphur-containing amino acid (31)

Soybean is a good source of oil and protein. The oil content of the soybean is highest among all the legumes and its protein content is again highest among all the cereals and legumes.it is a

good source of amino acids with very high in lysine and its composition is almost similar to the animal protein (38).It has other effects like lowering plasma cholesterol and triacylglycerol(Baba et al., 2004), preventing cancer (63), diabetes, and obesity, protecting healthy digestive tract, and protecting bone, and kidney from irritants (19).

the topmost producers i.e. almost 85%; of the world are are USA, Brazil, Argentina, India and China. These are prepared crushed annually for the production of soy meal and oil and then 98% is crushed for further animal feed. Soybean is having a lot of agricultural importance as many proteins and similar compounds can be isolated from that .those compounds may have some nutritional and/or toxic effect on the organisms.

Soybean lectin is a protein having ability to bind with the carbohydrate so called as the glycoprotein. They have high binding protein specificity to terminal non-reducing N-acetyl-D-galactosamine but less to D-galactose and play a lead role in recognition of *Rhizobium japonicumby* plant which helps in nitrogen-fixing symbiosis by allowing certain bacteria to work together with the roots of the soybean plant (6). They have important role in cell to cell communication (29). Sbl has two main functions, first they cause cell injury and death by acting upon the carbohydrate moieties present on the cell surface and when damage accumulates harmfully affects the gastrointestinal, immune, and other systems of humans and other animal species. Secondly they remain in a symbiotic relationship with the Rhizobium bacteria by fixing atmospheric nitrogen in the roots of the soybean plant (The Whole Soy Story). Reduction of SBL in normal diet may lead to the decrease in the trypsin activity in protein level and augment the amylase activity of amylase in the pancreatic juice (26).

SBL has the 60-90% affinity to bind with the bone marrow mononuclear cells, including mature myeloid, erythroid, and lymphoid cells they have almost no toxic effect to the human hematopoietic progenitor cells but having very low binding capacity .They bind non-covalently to the cell surface carbohydrate so shows agglutination activity. Moreover it also has the specificity towards certain tumor cells like neuroblastoma, breast cancer, and Burkitt's lymphoma cells which directs towards the multiple clinical applications of this protein. It has also many potential uses for bone-marrow transplantation (BMT) . These lectins have inhibitory effect on absorption of non-heme iron (55). Soybean from different plant products have variety

of property. Trypsin inhibitors are produced by the yellow soybean (40). Lectin of black soybean is stable up to 40^0c for 20 min., whereas small glossy soybean is stable up to 70^0c for 30 min. SBL helps in removal of cancer from the marrow and also decreases the risk of graft versus host disease. Again it is also used for purging of the bone marrow during the acute lymphoblastic leukemia treatment (Reisner Y, 1983). It increase the blood glucose level in both diabetic and non-diabetic rats (26) and also act as the inhibitor of the HIV-1 reverse transcriptase and breast cancer proliferation (48). Soybean also decreases the chances of breast and prostate cancer they reduces the protein digestion due to the presence of the protein inhibitor. Along with that it also induces the pancreatic tumors in some animals (24). Black soybean has mitogenic effect towards splenocytes than that of CON-A.it also imparts a immunomodulatory effect on the interleukins production (15).

Isoflavens have diversity of biological activities and decreases the risk of some chronic diseases and these are mainly found in the soybean. It has also some hypocholesteromic effect due to the effect of Isoflavens. Due to this Isoflavens it also helpful in case of several diseases like cancer, osteoporosis, cardiovascular disease and also alleviate menopausal symptoms. Postmenopausal women were observed of improving cognitive ability in after intake of soybean extract. The atrophy of the microvilli is caused by SBA which reduces the viability of the epithelial cells and the weight of small intestine increases because of hyperplasia of crypt cells (23; 51). Soybean gene is highly regulated by during life cycle as it represents one of the repertoires of seed protein genes. During soybean embryogenesis Lectin gene is extremely expressed at its highest level lectin mRNA constitutes 0.75% of the embryo mRNA mass (47). Thermal treatment of the soy lectin reduces the protease activity by eliminating the lipoxygenase and volatile compounds that induce undesirable flavor. Again Heating soy proteins above 70°C causes dissociation of their quaternary structures by denaturing its subunits, and promotes the formation of protein.

Raw soybeans contains Trypsin inhibitors cause growth inhibition, pancreatic hypertrophy and hyperplasia in experimental animals High amount of oligosaccharides are present in soybean having mainly of raffinose and stachyose, poorly digested and have been concerned as causes for the reduced utilization of energy from soybean meal fed to poultry (35). Mitogenic effect of soybean observed under any physical or chemical basis (41) SBL reacts with the spleen and cyclo-phosphamide generated suppressor cells can thus be isolated by agglutination from the

bone marrow (28). Soybean lectin reportedly reacts preferentially with some rumen fungi (3). Lectins purified showed effects with all the blood groups of human being (4). It reacts against antibodies (22). Haemagglutination shows the activity of the lectin (46). The haemagglutination activity varies in different soybeans. Soybean lectin mediated agglutination of sheep red blood cell (SRBC) shows differential effects on human marrow cell suspensions (52).

Anti-cancerous property of soybean lectin

It was for the first time reported that soybean agglutinin was capable of inhibiting the growth of tumor in rats (34). Pattern of lectin binding on the ell surface suggests about the type of malignancy of the tumor (54). These are also versatile markers, help in studies of various histochemical, biochemical, and functional methods for cancer cell classification (45). The exact mechanism is still not clear but lots of pathways are proposed for this. This has been found that targeting the surface lectins present on the tumor cells can be helpful for the treatment of cancer. Treatment with anti-lectin antibodies can help in suppressing the tumor growth and colonization (59). These can be used for studying the metastatic spreading pattern and the prediction of lymphatic attack.(8).these can also be used as the carrier for targeted drug delivery. Soybean lectin is useful for analytical indicator of stomach cancer (56).

Soybean agglutinin related with the recognition of the human lymphoma cells where sialylation of particular carbohydrate residues is associated with the spontaneously metastatic capacity of human lymphoma cell lines.(1). The number of macrophages increase with the SBL treated cells over the control (21) Another study focuses on the human bone marrow cells which were mixed with the neuroblastoma cells from different cell line and then mixtures are separated by the SBL where all the neuroblastoma cells expressed the presence of receptor for SBL. By targeting SBA-bound toxins, Improved purging of neuroblastoma cells was achieved in another test Burkitt's lymphoma cells were purged where it showed the similar results (44). It shows its effect upon the transplants of leukemia patients (10) another mechanism called the Cytoagglutination or aggregation by which the interaction of cancer cells can be detected. (30).

Table-1 Soybean Inhibitory Effects On Malignant Cells (16)

TYPES OF TUMOR CELLS	EFFECT
T-cell	No increase in the reaction and graft failure
Neuroblastoma, breast cancer, Burkitt's lymphoma cells	attaches excellently to accessory cells without binding the human stem cells
Dalton's lymphoma, peripheral blood lymphocytes,macrophages	destroy tumor growth
human colon cancer	Cytoagglutination/aggregation
myeloma	Cytoagglutination/aggregation
Prostatic tumors	no difference between prostatic hyperplasia and adenocarcinoma
T-cell leukemia, B-cell	Interaction of Leukemia of SBA with the tumor cells is not related to the cell cycle
Human gastric carcinomas	Direct contact/adhesion/binding to cell membrane/ cell membrane receptors
Burkitt's lymphoma cell	SBA lectin–binding sites are masked by sialic acid

There are many bioactive peptides which can be derived from soybean protein which are having many effects on health and can be used for age-related chronic disorders, such as cardiovascular disease, obesity, decrease immune function and cancer. In contrast to most small-molecule drugs, peptides have high affinity, strong specificity for targets, low toxicity and good penetration of tissues (5).mechanism of action of lectin involves effect on tumoral cell membranes, the reduction in cell proliferation, the induction of tumor-specific cytotoxicity of macrophages and the induction of apoptosis. Lectins have also a strong interference on the immune system by producing the interleukins (15).

Structure of Soybean Lectin

Soybean lectin has the specificity for N-acetyl-D-galactosamine and D-galactose (37;42). It is tetrameric structure having four subunits of 30 Kda and each having capacity to bind with one

carbohydrate per subunit (14). Ca^{+2} and a transition metal mainly regulates the sugar binding capacity of the SBL.As SBL is a glycoprotein so per subunit it has four oligosaccharide moieties (Man_9 ($Gl_cNA_c)_2$) (36). When the amino terminus of 120 residues are aligned with the lectin if concanavalin A ,it showed extensive homology thus signifying a circular permutation of the sequence (27).

The soybean lectin has a tetramer structure and having two canonical dimer. Here interaction occurs between the two curved 12 stranded back sheets and the outermost strands, resulting a large channel type structure on the middle of the tetramer, which is having disordered C-terminus of these lectins and it protects the protein against the proteolytic degradation. This kind of formation made by the outermost strands consists of mainly no. of comparatively short side chains that that interpolate in a zipper-like fashion. (39)legume lectins mainly contain two sides basically i.e. carbohydrate binding site and hydrophobic ligands(13; 12; 20).there is presence of a binding site per tetramer (9). There is presence of Ca^{+2} and Mn^{+2} per subunit and each of the subunit has the power to bind with the sugar moieties independently (58). It also forms dimer of dimers. The dimerization process takes place by antiparallel side-by-side alignment of two flat six-stranded ‘‘back’’ β-sheets, one from each monomer, giving rise to the formation of a contiguous 12-stranded sheet forming the canonical dimer. these dimers attached in a back to back fashionto form the tetramer.

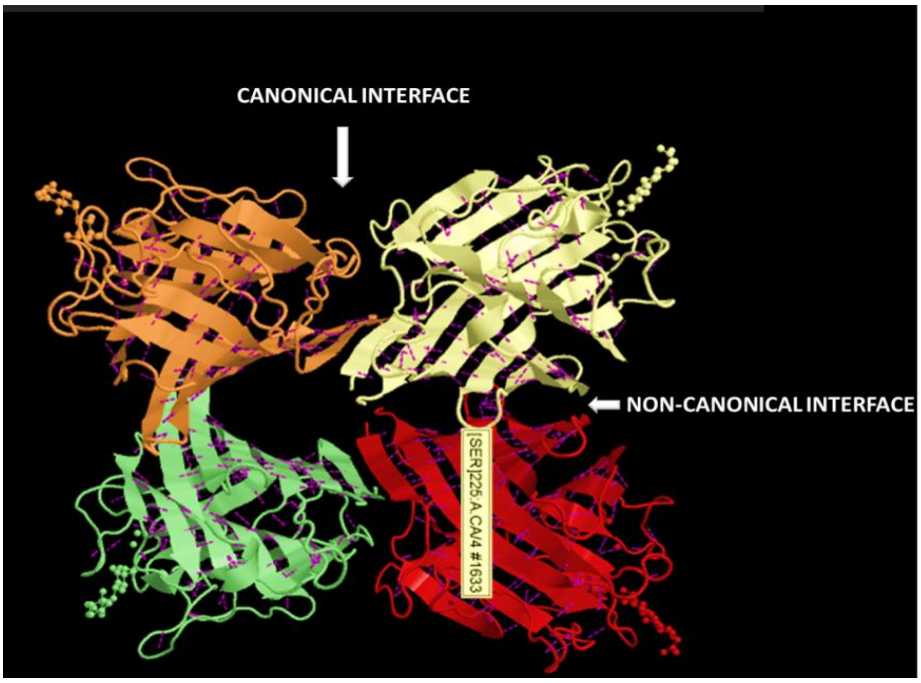


Fig 1 PDB structure of soybean lectin

So it has four interfaces in which two are canonical and other two are non-canonical.it has been found that SBL tetramer has more structural stability than that of the ConA. Analysis of the isothermal denaturation data gives the idea about the conformational stability of SBL. Unfolding pattern was observed by the guanidine chloride induced denaturation where it shows a reversible two state unfolding mechanism.

This conformational stability is due to the subunit interaction pattern of the protein. Ionic interaction of the non-canonical interface of the protein plays an important role in the structural stability. the sugar chain of the SBL present in the non-canonical sub unit and it is found to interact with the amino acid residues in the adjacent non-canonical subunit which helps in stabilization (58).

SOYBEAN – A BRIEF DESCRIPTION

Table 2 Taxonomic classification of Soybean

Classification	
Kingdom:	Plantae
Order:	Fabales
Family:	Fabaceae
Subfamily:	Faboideae
Tribe:	Phaseoleae
Subtribe:	Glycininae
Genus:	Glycine
Species:	max
Common name	soybean

Fig 2 soybean plant and fruits

Fig 3 Soybean seeds

Objective

- **Isolation and of soybean lectin**
- **Characterization of soybean lectin**
- **Understanding the anti-cancerous activity of soybean lectin**

MATERIALS AND METHODS:

CHEMICALS:

Sodium hydroxide (NaOH), Sodium carbonate (Na2CO3), glycine,Cupper sulphate(CuSO4) , Potassium sodium tartarate (KNaC4H4O6) were purchased from SRL, Sisco Research laboratories Pvt. Ltd., Mumbai. Acrylamide, bisacrylamide, Ammonium persulphate (APS), Sodium dodecyl sulphate (SDS), N,N,N‘,N‘-tetramethyl enediamine(TEMED), Bovine serum albumin(BSA), Tris were purchased from Sigma Aldrich, USA. Folin-Ciocalteau phenol reagent, Potassium Dihydrogen Phosphate (KH2PO4), Potassium hydrogen phosphate (K2HPO4) were purchased from S.D. fine chem. Ltd., Mumbai. Acetic acid, Bromophenol blue, agarose were purchased from Himedia, Mumbai. Glycerol was purchased from RANKEM Pvt Ltd. Ethanol purchased from MERCK Chemicals, India. Pre stained molecular weight marker was purchased from Bio-Rad, India. Methanol, Silver nitrate, Sodium thiosulphate were purchased from sigma Aldrich.

SAMPLE COLLECTION:

The Soybean (Glycine max) seeds were collected from deep forest of Anugul, Odisha. The chemical used in the study are supplied by Hi-media and Sigma Aldrich.

PREPARATION OF LACTAMYL SEPHAROSE 4B AFFINITY MATRIX:

EPOXY ACTIVATION OF SEPHAROSE 4B:

8gm of lactamyl sepharose 4B matrix was washed with 12ml distill water and mixed with 5.2ml of 2N NaOH and 1.3ml epichlorohydrin were added so that the final concentration of the various components were 30% v/v sepharose, 5% epichlorohydrin, 0.4 M NaOH. It was cover with aluminum foil and incubated at 400c for 2h with shaking. It was then transferred to a glass filter funnel and the gel was washed with 500 ml of distilled water.

PREPARATION OF AMINO SEPHAROSE 4B:

Epoxy activated sepharose 4B was suspended in 1.5 volume of concentrated ammonia solution i.e. 12 ml. The suspension was incubated at 40^0c for one and half hour. It was then again transferred to a glass filter funnel and the gel was washed with distilled water.

COUPLING OF LACTOSE WITH AMINO SEPHAROSE 4B:

8gms of Suction dried amino sepharose 4B was suspended in 6ml of 0.2M K_2HPo_4 buffer, which was containing 102mg NaCNBH3 and 208mg of Lactose. The Suspension was incubated at room temperature for 10 days with occasionally shaking. The free amino groups which remained in the gel were acetyled by adding 2 ml of acetic anhydride. The suspension was incubated in the room temperature for 1 hour. The lactamyl sepharose 4B thus obtained was subcequently washed with distilled water, 0.1 M NaOH, distilled water and 10 mM PBS subsequently. It was stored in distilled water with traces of sodium azide at 4^0C.

FILLING UP OF MATRIX AND STORAGE OF COLUMN

The column was taken and washed with MQ water and then with PBS. Matrix was poured inside the column slowly that precaution should be taken to avoid air bubbles buble in the matrix.then PBS was continuously supplied til the matrix has set completely and formed a dense layer of it.

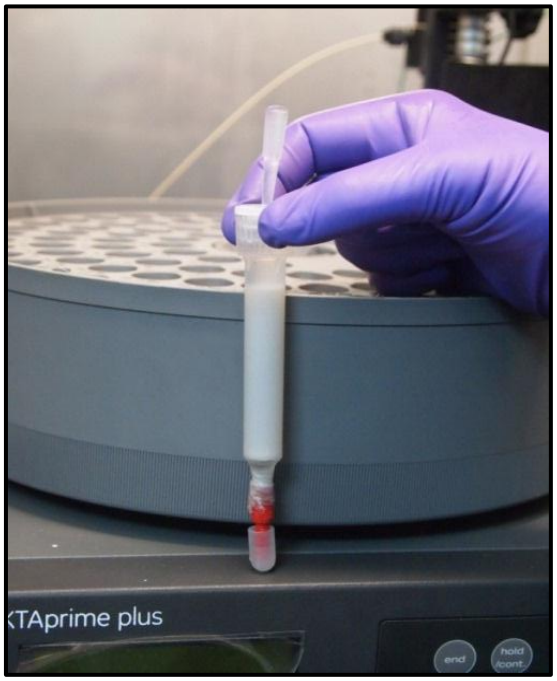


Fig. 4 Lactamyl Sepharose 4B Column

ISOLATION OF LECTIN FROM SOYBEAN SEEDS

SEED COAT REMOVAL:

100 gms of Soybean seeds were taken and grinded in mixer for decortication The uncoated seeds were soaked in PBS for at least 24hrs. The seed pastes were collected in 500 ml centrifuge bottles by grinding the seeds with PBS. centrifuged was done at 10,000 rpm, 4^0c for 20 mins. and Supernatant was collected. Some volume of supernatant were stored at 4^0c for protein estimation and Haemagglutination assay and the remaining were taken for salting out Process.

SALTING OUT:

Protein are having multiple charged groups, the concentrations of dissolved salts decides its solubility, the polarity of the solvent, the Ph, and the temperature. By selectively precipitate certain proteins some or all of these variables can be manipulated where as others remain soluble. The solubility of a protein at high ion concentrations decreases as salt, particularly sulphate salts; is added, a phenomenon called "salting out". It is the basis of one of most commonly used protein purification procedures Since proteins precipitate at different salt concentrations. Many unwanted proteins from the solution eliminated by adjusting the salt concentration in a solution containing a mixture of proteins to just below the precipitation point of the protein to be purified. This procedure results in a significant purification and concentration of large quantities of protein. Ammonium sulfate, (NH4)2SO4 , is the most commonly used salt for salting out proteins because its large solubility in water, its relative freedom from temperature effects, and it has no harmful effects on most of the proteins. The most effective pH region for salting out of the desired protein is at its isoelectric point because the protein is least soluble when its net charge is zero.

Collected Crude supernatant of soybean was taken for 20% cut off. According to the salt chart ammonium sulphate was measured and thoroughly grinded by mortar pestle to make a soft amorphous salt. Then it was added to supernatant pinch wise with a continuous stirring`

Fig 5 Crude supernatant of soybean for 20% cut off

After addition of the salt the sample was stored in 4^{o}c for overnight incubation. Next day the sample was centrifuged and supernatant was collected again for the next cut off i.e. 60% cut off. Supernatant collected, was measured and salt is added according to the salt chart. Similar procedure was followed for 90% cut off. After incubation of 90% cut off pellet was collected and dissolved in minimum volume of PBS and prepared for dialysis.

DIALYSIS

One of the common operations in biochemistry and protein biology is the dialysis which helps in separating dissolved molecule by passing through the semi-permeable membrane according to their molecular dimensions.

Semi-permeable membrane is containing pores of less than macromolecular dimensions. These pores allow small molecules, such as those of solvents, salts, and small metabolites, to diffuse across the membrane but block the passage of larger molecules. Cellophane (cellulose acetate) is the most commonly used dialysis material although many other substances such as nitrocellulose and collodion are similarity employed. So, dialysis is a method in which an aqueous solution containing both macromolecules and very small molecules which are placed in a dialysis bag which is in turn placed in a large container of a given buffer or distilled water. Thus small solute

molecules freely pass through the membrane, and after several hours of stirring the equilibrium will reach (the concentration inside and outside the bag are the same). Thus, at equilibrium the concentration of small molecules outside and inside the bag is the same while the macromolecules remain inside the bag. During dialysis the external fluid should be changed in order to reach the required composition inside the dialysis bag. There are three factors that affecting the rate of dialysis: the first is the concentration differences of that molecules between the internal and external solution (which is the driving force for the movement of the molecules). The second is mixing on both sides of dialysis membrane will increase the rate of movement prevent the small particles on the side of low concentration. The third is dialyzable particles size versus pore size of the membrane, substances that are very much smaller than the pore size will reach equilibrium faster than substances that are only slightly smaller than the pores. The main point to be noted is that there is a rapid initial drop in dialysis process followed by a slow approach to equilibrium.

Pellet dissolved in PBS after 90% cut off was taken for dialysis. it was kept in dialysis for 3-4 days. First days the sample was dialyzed with water then rest of the days with PBS. It should be taken care of that the dialysis bag filled with the sample should completely be dipped in PBS.

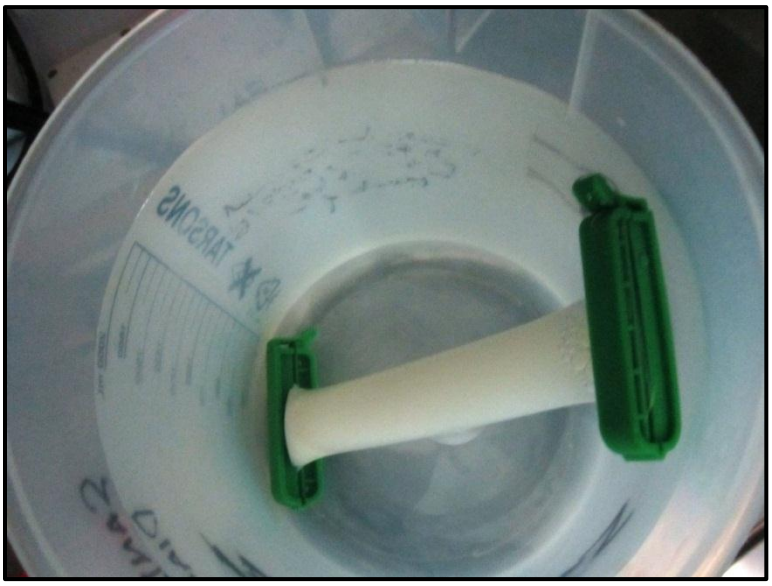


Fig 6 Dialysis of 90% cut off sample

After completion of dialysis the sample was centrifuged at 10000 rpm, 4^0c for 30 mins. Then the supernatant was collected and it as store in -20^0c affinity chromatography of the sample.

AFFINITY CHROMATOGRAPHY

It is technique by which proteins are purified those have the ability to non-covalently and reversibly bind specific molecules, known as ligands. in the classical chromatography techniques protein is purified on the basis of a unique biochemical property. here the ligand is covalently attached to a matrix, which must be chemically inert, porous, and have a variety of functional groups suitable for coupling with diverse ligands. Ligands are used in affinity chromatography, depending on the protein to be purified (61) . A particular affinity chromatography technique, which uses carbohydrate adsorbents (ligands or matrices) for purification of glycan-binding proteins or lectins, is called carbohydrate affinity chromatography. interactions of multivalent lectins with complex, branched carbohydrates containing multiple epitopes, result in high-avidity binding with nanomolar or even picomolar dissociation constants. lectins bind carbohydrates non-covalently and reversibly and at least the most widely used ligands, mono- and disaccharides, are usually bound relatively weakly, so that the lectin is readily released from an affinity column by competitive elution using specific free carbohydrates.(60).

The collected and stored dialyzed sample was now taken for affinity chromatography by Akta prime using previously prepared lactamyl sepharose 4B column. The lactamyl sepharose 4B column was washed by PBS solution (pH7.2) and stabilized. Then after PBS wash sample was loaded after finishing the sample the column was again washed with PBS and then the protein was eluted with 10 ml of 1M of lactose solution. The collected protein was immediately stored at 4^0c.

Fig 7 Lactamyl Sepharose 4B column fitted with Akta prime for affinity chromatography

DIALYSIS:

The purified affinity sample stored at 4^0c,was dialyzed again in water for 1 day .

Fig. 8 Dialysis of 90% cut off affinity solution

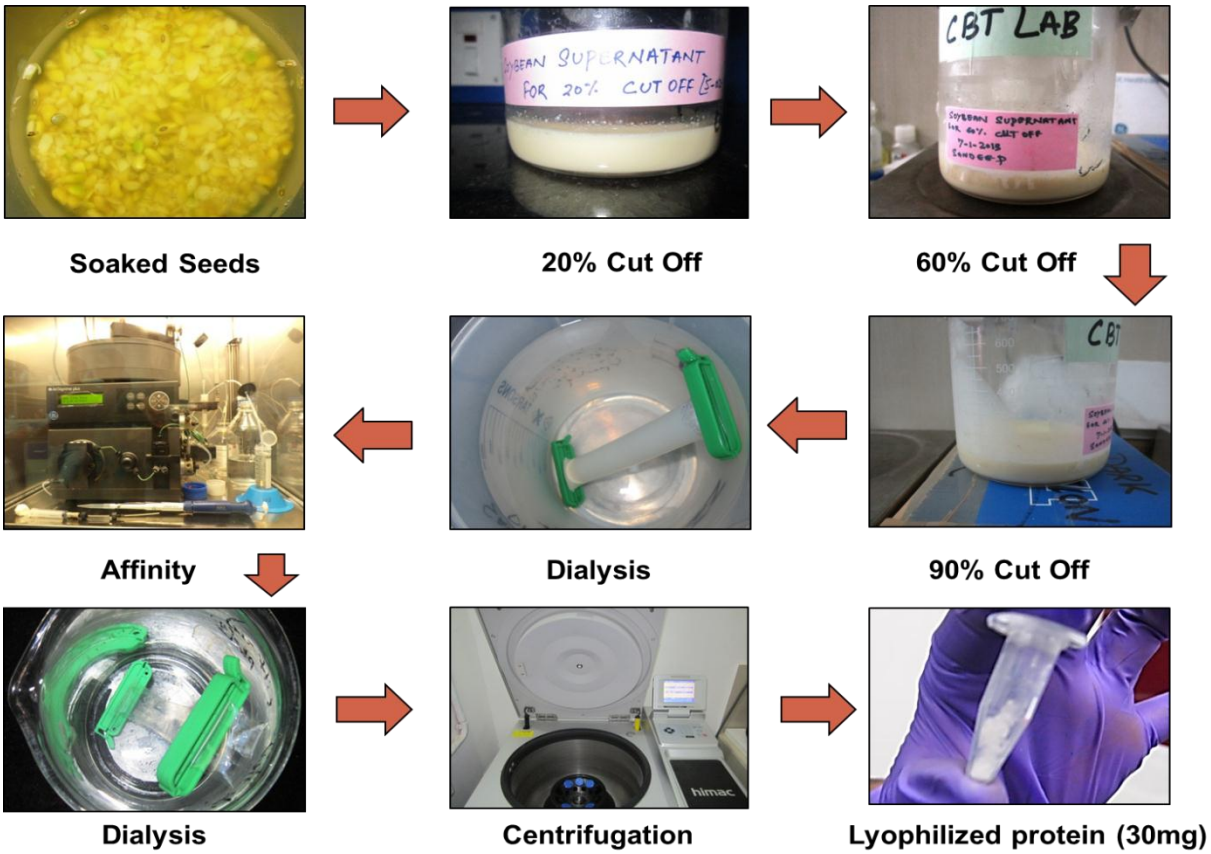


Fig 9 Pictorial representation of complete process of isolation of SBL

DETERMINATION OF CONCENTRATION OF PROTEIN

The concentration of crude, 20% cut off, 60% cut off, 90% cut off and 90% cut off affinity were measured by Lowry et.al (43) using bovine serum albumin as the standard protein

Reagents required:

1. BSA stock solution (1 mg/ml)
2. Analytical Reagents

A- 50 ml of 20% sodium carbonate mixed with 50ml of 0.1N naoh solution(0.4gm in 100ml of distilled water)

B- 10 ml of 1.56% CuSO4 solution made with 10ml of 2.37% sodium potassium tartrate solution.prepare analytical reagents by mixing 2ml of B with 100 ml of A

3. Folin's reagent-ciocalteau reagent solution (1N) dilute commercial reagent (2N) with as equal volume of water on the day of use(2ml distilled water and 2 ml of reagent)

Different dilutions of BSA stock solutions were prepaed.0.2 ml of protein sample was taken to which 2ml of alkaline cuso4 was added to it and mixed than kept of 10mins incubation. Ten 0.2 ml of Folin's reagent was added to it and incubated for 3o mins. in dark. Then O.D was taken at 600nm. The graph was plotted for determining the unknown concentration of the protein of interest taking absorbance in Y-axis and concentration in X-axis.

HAEMAGGLUTINATION ASSAY

PRINCIPLE:

Lectins have the property to agglutinate the erythrocytes. Soybean lectin forms a mesh like structure in between the blood erythrocytes which do not let the blood to be clumped. More the concentration of lectin agglutination will be more.

PROCEDURE:

Collection of blood

Human blood was collected from common welfare society hospital, Rourkela in a 15 ml falcon tube and EDTA was added to it.

Preparation of human erythrocyte for assay

1 ml of blood was taken and centrifuged at 1000rpm for 5mins in room temperature. Serum was discarded and pellet was collected 10 ml of PBS was added to it for washing and centrifuged again for 5 mins at 1000rpm in room tempreture.by discarding all the pbs 100µl of blood pellet was collected and mixed with 10 ml of PBS. This is the final blood sample for the assay.

The assay was carried out in 96 well round bottom microtitre plate. The first well of each row was served as positive control and the last well served as negative control since it contain 100µl of blood and 100µl of PBS solution.in the first well normalized protein samples were added according to their volume calculated. Then each well was given with 100µl of PBS and the protein sample was serially diluted till the negative control. Then 100µl of prepared blood was added to each of the well.it should be taken care of that the final volume of each well should be 200µl.

ELECTROPHORESIS

Electrophoresis of macromolecules is one of the most common methods for the analysis of these molecules. Electrophoresis means movement (phoresis) in an electric field (Electro). Complex mixtures of either proteins or nucleic acids (RNA or DNA) can be separated by electrophoresis.

SDS PAGE

The molecular mass of the subunits was determined by SDS page (8%) The poly acrylamide gel electrophoresis was done according to the protocol given in the Book―Molecular cloning‖ by Sambrook & Russell. The mixture of 30µl of sample, 10µl of sample loading buffer was added to the well.

NATIVE PAGE

Native page was done to determine the molecular mass of the protein (8%).

SILVER STAINING

Silver staining was done to make the bands visible.

MTT ASSAY:

Principle

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The internal environment of proliferating cells is more reduced than one of nonviable cells. This formazan was later dissolved by using any organic Solvent(DMSO) which imparts purple colour.

Procedure:

The cells were re harvested in T_{25} culture flask (BD) and trypsinised when they reached at 80-90% confluency. The cells were seeded in 96 well plates (TARSON). After 24 hrs. lectins were treated and kept for 72 hrs and then MTT WAS added and kept about 4 hrs. After that DMSO(SIGMA) was added to dissolve the formazan and O.D was taken at 562nm in a Elisa plate reader(Perkin Elmer).

TRYPAN BLUE EXCLUSION ASSAY

PRINCIPLE:

The trypan blue exclusion test is a rapid method to assess cell viability in response to environmental insults. It is simple and inexpensive. The dye exclusion test is based on the ability of viable cells to be impermeable to trypan blue, naphthalene black, erythrosine, and other dyes. When membrane integrity of the cells is compromised, there is uptake of the dye into the cells so that viable cells, which are unstained, appear clear with a refractile ring around them and nonviable cells appear dark blue colored with no refractile ring around them. The following method is for trypan blue, the dye used most commonly.

PROCEDURE:

Drug treated cells were seeded on a 6 well plate then trypan blue was added and kept for 6-7 hrs. Then it was seen under the fluorescence microscope (Olympus Fluorescence microscope IX 70)

DAPI STAINING

The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove.1 Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. It mainly shows the chromatin condensation during apoptosis in nucleus.

Procedure:-

Drug treated preseeded cells are treated with4% paraformaldehyde and then washed with PBS. Then DAPI stain was added to it and incubated for 5 mins. and observed under florescence microscope.

DNA FRAGMENTATION ASSAY:-

One of the most easily measured features of apoptotic cells is the break-up of genomic DNA by cellular nucleases. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3'-hydroxyl termini in the DNA. This property can be used to identify apoptotic cells by labeling the 3'-hydroxyl ends with directly conjugated fluorescein-deoxyuridine triphosphate nucleotides (FITC-dUTP).

CLONOGENIC ASSAY

Clonogenic assay or colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo “unlimited” division. Drug was treated to the colony of cells and survival was checked according to the dose dependent manner.

Procedure-

Pre-seeded cells were treated with Sbl. After rinsing with fresh medium then it was let to grow for 14 days. Then crystal violet was added and no. of colonies was counted.

RESULTS

Isolation and purification of SBL

Soybean lectin was purified by affinity chromatography by Lactamyl Sepharose-4B column.

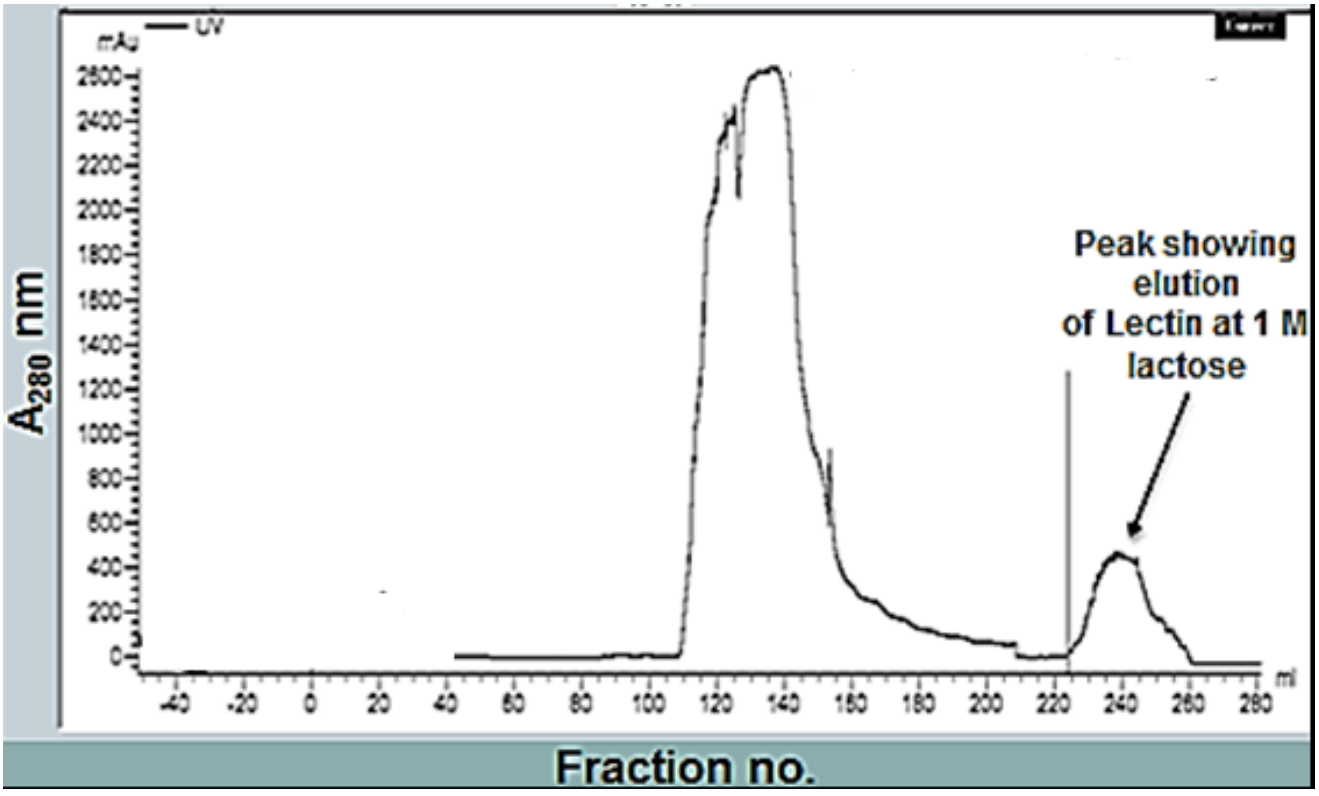


Fig. 10 Elution profile of soybean lectin by Affinity chromatography

Determination of protein concentration

Determination of the concentration of the proteins (crude, 20% cut, 60% cut, 90% cut, 90%affnity, 90% affinity dialysed) were done by Lowry method which is depicted in Table 2.

Table 3 Concentration of proteins

Sample	Concentration(mg/ml)
Crude supernatant	98.898
Crude pellet	100.55
20% cut off supernatant	62.48
20% cut off pellet	75.26
60% cut off supernatant	81.94
60% cut off pellet	31.38
90% cut off supernatant	6.10
90% cut off pellet	13.07
90% cut off dialysed	0.464
90% cut off affinity dialysed	0.555

HAEMAGGLUTINATION ASSAY:

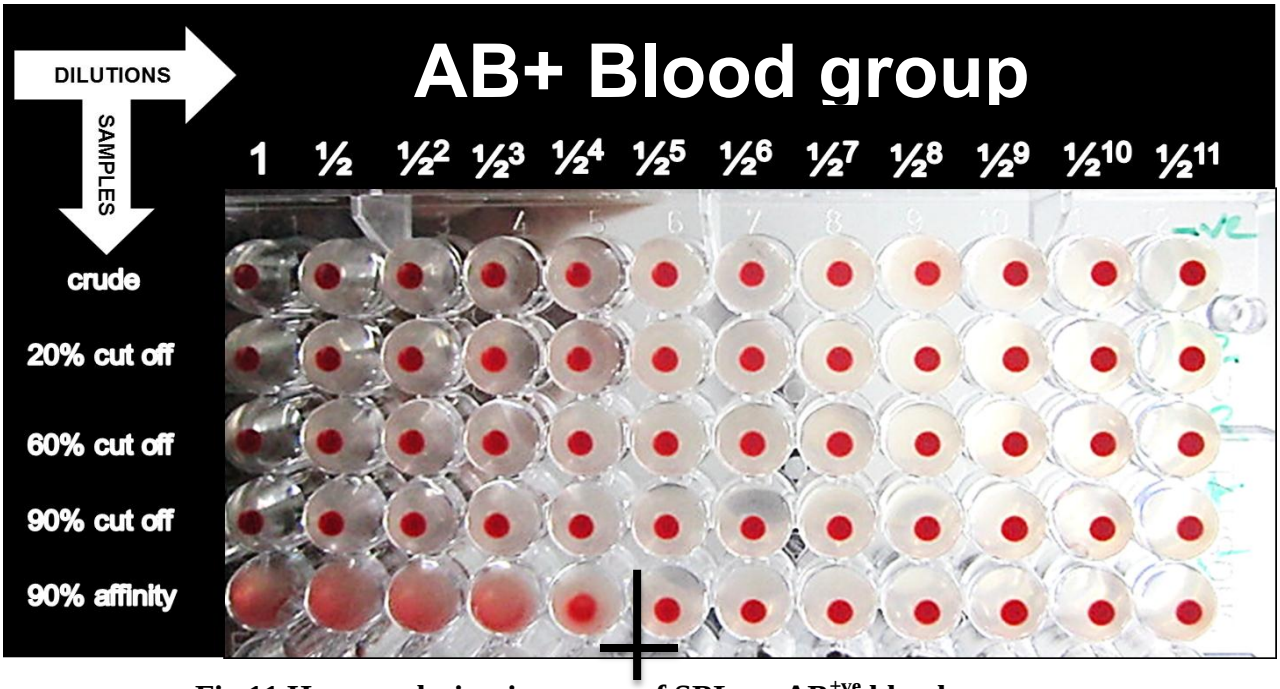


Fig 11 Haemagglutination assay of SBL on AB^{+ve} blood group

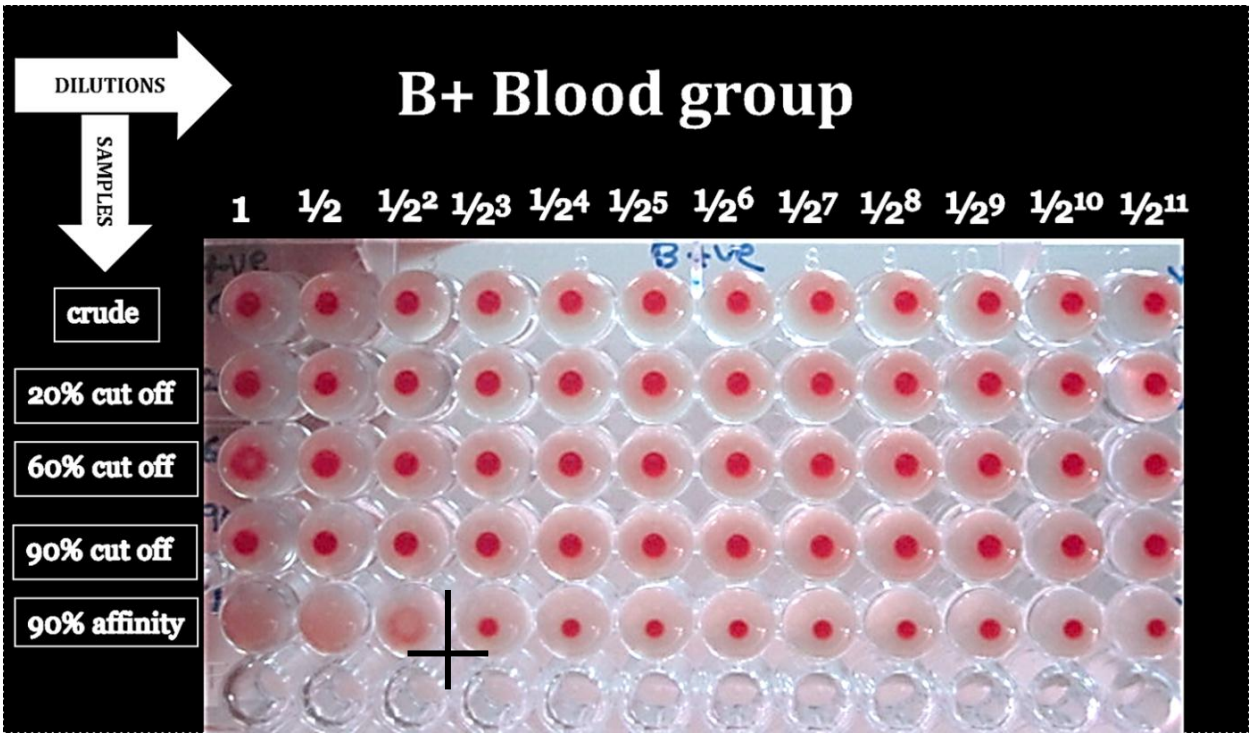


Fig 12 Haemagglutination assay of SBL on B^{+ve} blood group

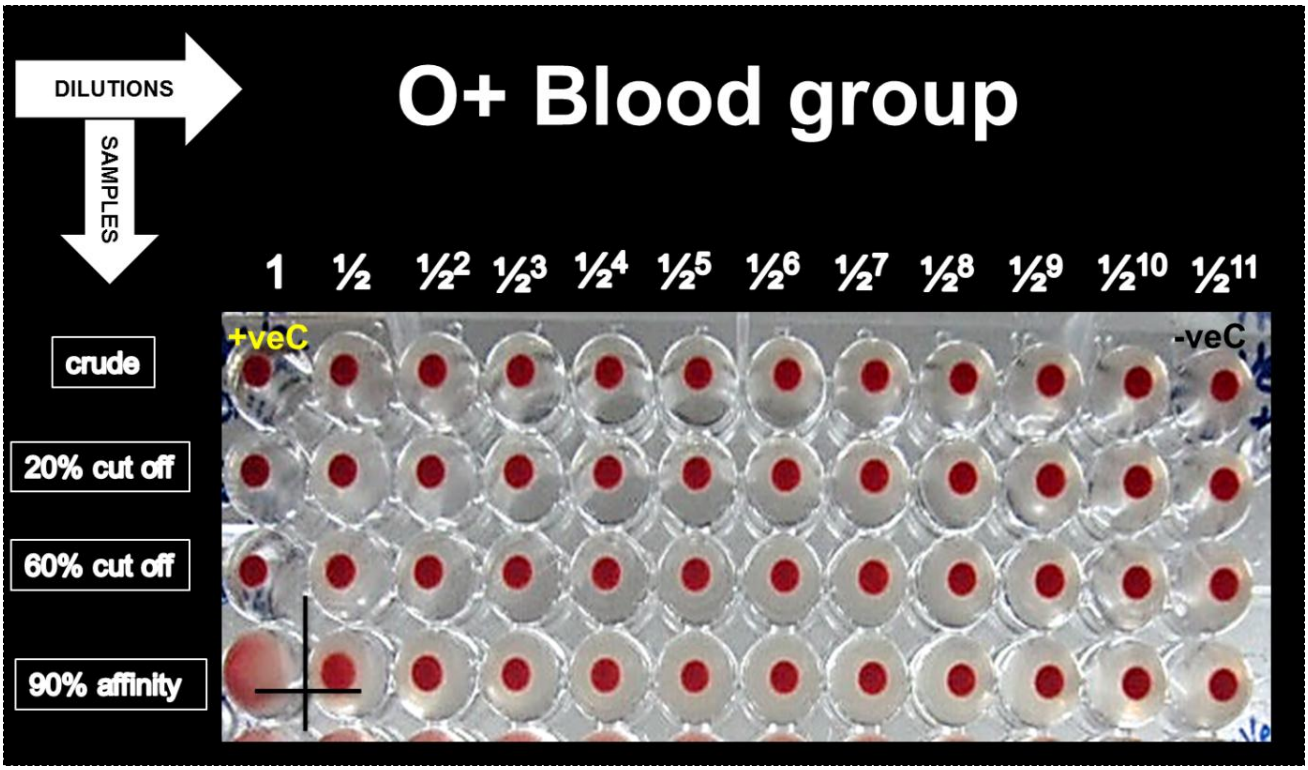


Fig 13 Haemagglutination assay of SBL on O+ve blood group

Table 4 Titer value of the blood groups

Blood group	Titer value
AB+ve	16
B+ve	4
O+ve	1

SDS-PAGE

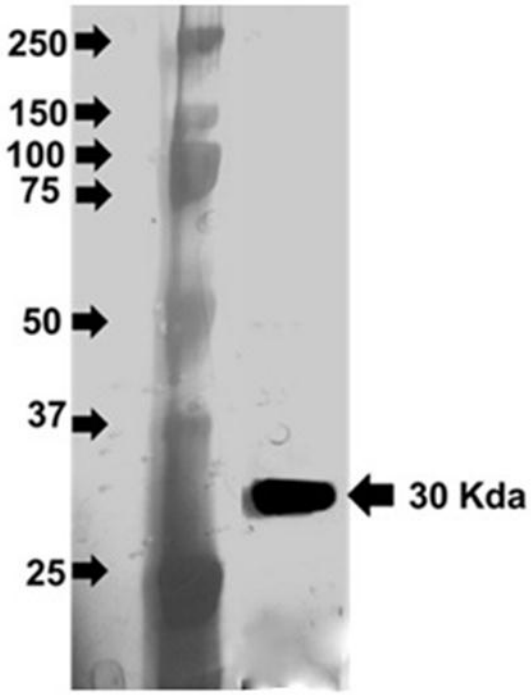


Fig 14 SDS PAGE 8% electrphoretogram

NATIVE PAGE

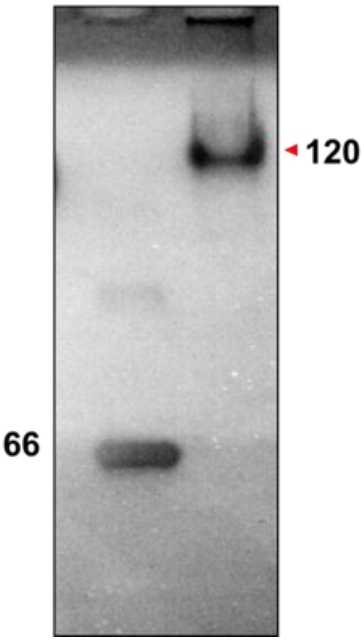


Fig 15 Native Page (8%) electrophoretogram

MTT ASSAY

Cells were treated with SBL, and then cell viability was measured by MTT assay. SBL reduces the viability of HeLa and Hep 2 at indicated concentrations with six replicates in each concentration.

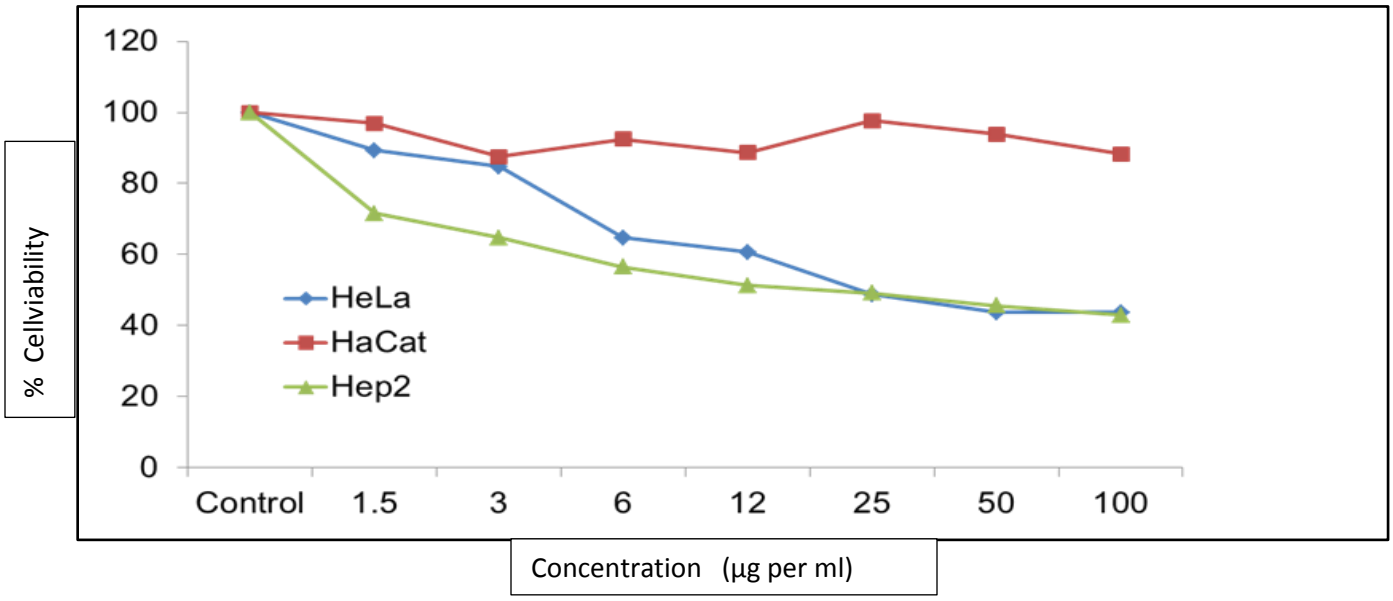


Fig 16 Dose dependent decrease in cell viability of HeLa, HaCat, Hep 2 cell lines

TRYPAN BLUE EXCLUSION ASSAY

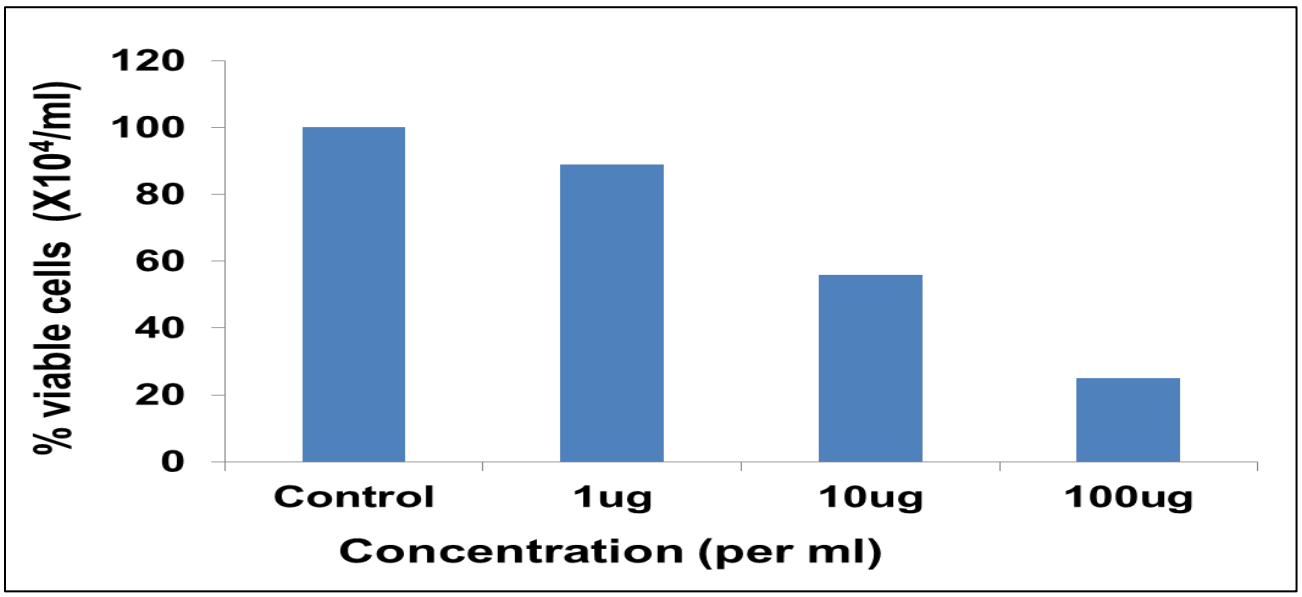


Fig 17 Trypan blue exclusion assay showing the Dose dependent decrease in cell viability

DAPI Staining

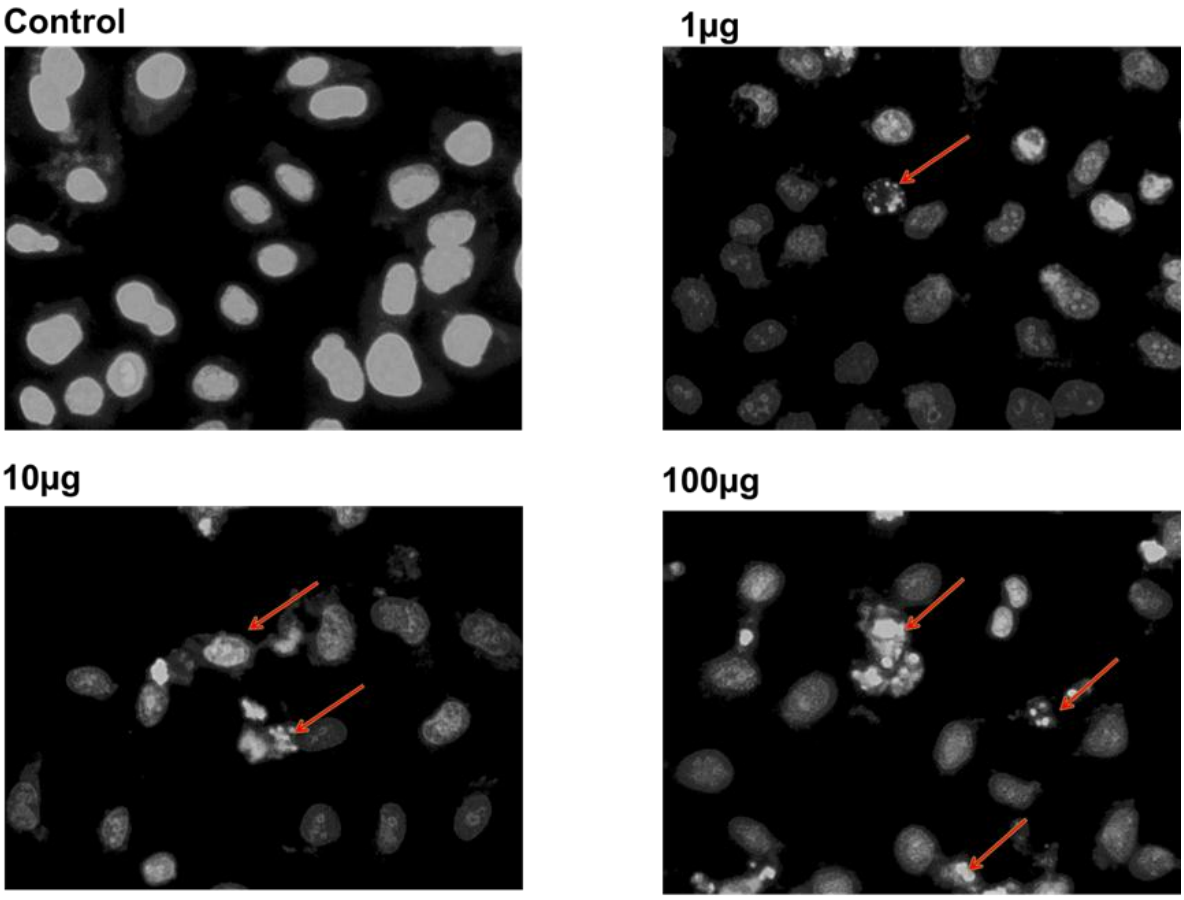


Fig 18- Study of apoptosis by morphological changes in nuclei of SBL treated HeLa cells by DAPI staining

Inference

Dose dependent effect on the nucleus of the cancer cell can be seen. Small autophagic bodies are forming due to chromatin condensation and DNA fragmentation

CLONOGENIC ASSAY

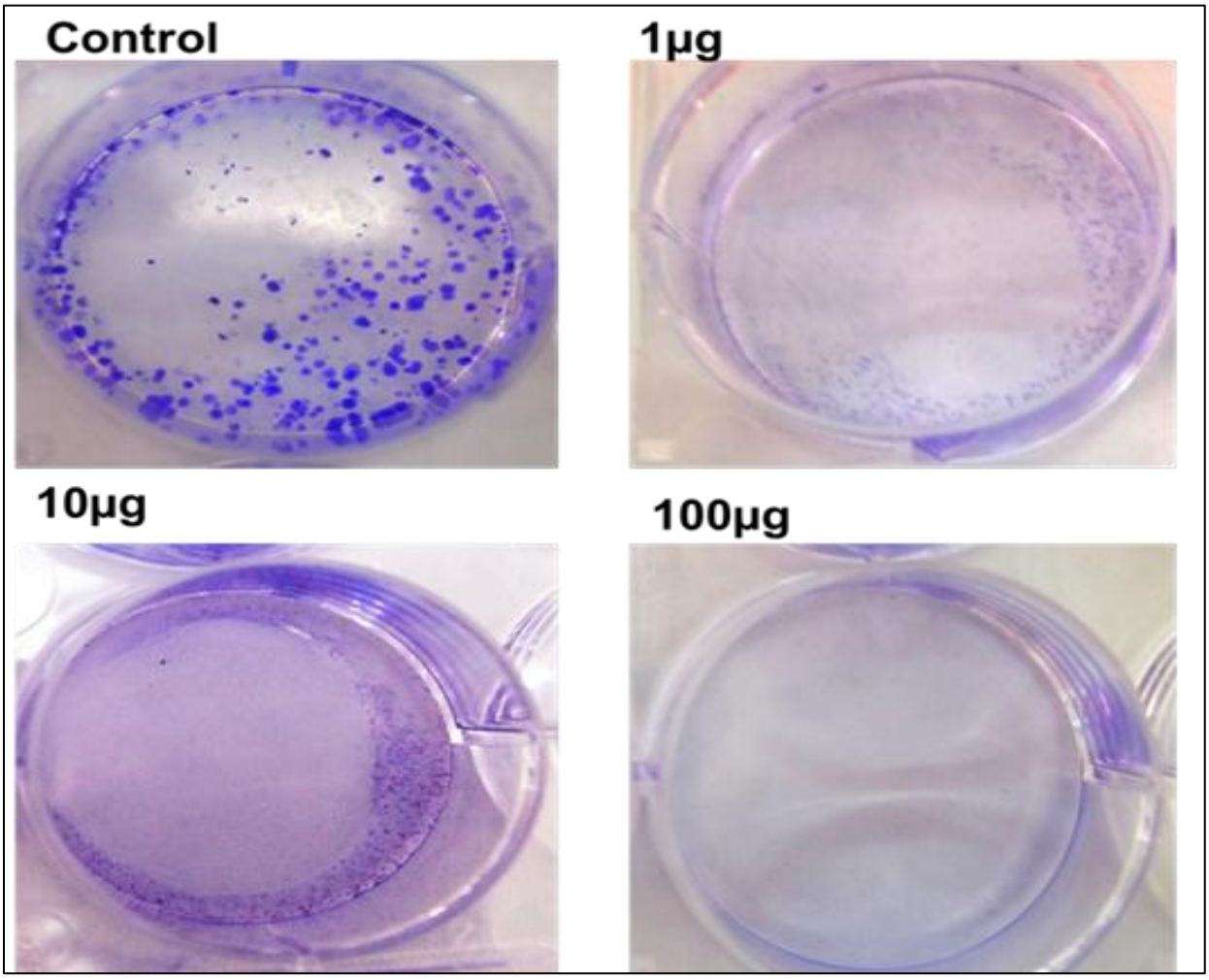
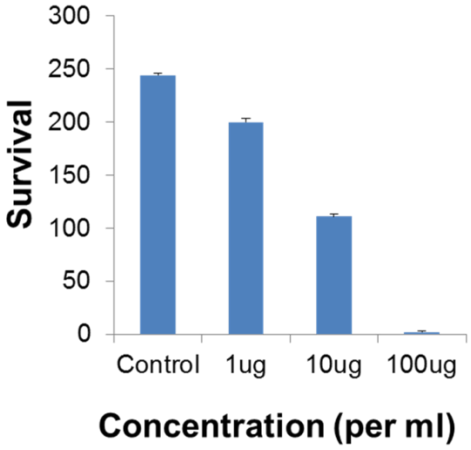


Fig 19- Effects of SBL on colony formation of HeLa cells



The colony forming assay was performed to examine the effects of SBL on colony forming ability of HeLa cells. A dose dependent colony forming inhibition effect was observed

Fig 20– Graph show ing no. of viable cells

DNA FRAGMENATATION ASSAY

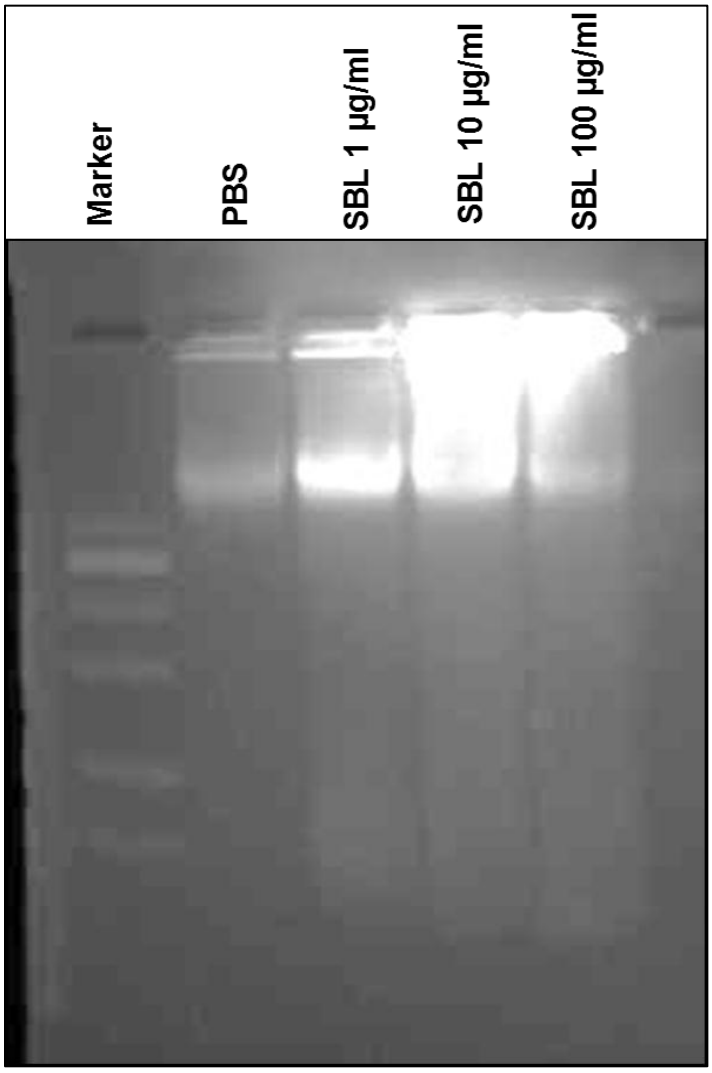


Fig - SBL induced DNA fragmentation in HeLa cells

Final step of apoptosis is the internucleosomal degradation of DNA, with appearance of DNA ladders due to activation of nuclear endonucleases considered a hallmark in most cells undergoing apoptosis. Typical DNA laddering was observed after 24 h exposure when the HeLa cells were exposed to various concentrations, of SBL indicating the occurrence of apoptosis at these concentrations

DISCUSSION

Treatment of soybean lectin on the cancer cells is an approach to study the effect. The important part of the project was to isolate the lectin and elucidating its function. Soybean lectin was isolated from the seeds by affinity chromatography using CNBr activated Sepharose 6B column (37) in the present study lectin was isolated successfully by using Lactamyl Sepharose 4B column which is a novel approach of isolating lectin from soybean. Single peak of affinity chromatography shows the positive result of isolation of protein.

Tto check the efficacy of lectin the isolated protein was treated with three blood groups(AB+, B+, O+). in three blood groups titer value found to be 16, 4 and 1 respectively. Thus sample isolated contains the protein of interest and which binds with the erythrocytes. This shows the carbohydrate binding properties which is determined by this assay. According to (67) the purified soybean lectin was treated with SDS and educing conditions in 2-β, mercaptoethanol. In the present study both denaturing and native polyacrylamide gel was done to determine the molecular weight of the protein. It is found to be 30 Kda by SDS Page and in native gel it was 120kda.

Cytotoxic effect of lectin can be seen by MTT assay on cancer cells (38) in the present study the same effect is seen with soybean lectin treated on the HeLa, Hep 2 and HaCat cells where there is very less effect on normal cell lines and the cancer cells were dying in a dose dependent manner. The viability of cancer cells can be checked by trypan blue dye exclusion assay (53). In the present study dose dependent decrease in the viability of HeLa cells can be seen.

Report says that Soybean lectin imped the proliferation of cancer cells (68). In the present study it has been shown that Sbl has property to inhibit the proliferation of cancer cells.it has also been reported that soybean lectin induce apoptosis in cancer cells(38)in the presented study DAPI staining and DNA laddering assays shows the basic properties of apoptosis that is DNA fragmentation and chromatin condensation.

Cervical cancer is one of the most prevalent cancers in India and HeLa cells are known to be the standard model of cervical cancer.so work was carried

Finally use of Lactamyl Sepharose 4B column for eluting lectin from soybean is a novel approach of isolation. Sbl is found to be effective on cancer cell. Further work can be done on the pathway study of the protein where Sbl induce apoptosis and autophagy(38).

CONCLUSION

From the above experiments performed it can be concluded that Sbl is successfully isolated and purified from the seed of Soybean (*Glycine max*) by affinity chromatography using Lactamyl Sepharose column 4B. SBL is characterized by Haemagglutination assay which shows that these proteins could agglutinate with the human erythrocytes due to the presence of carbohydrate-binding site. SDS page and native page gave the molecular weight of the protein and confirmatory result of presence of protein. Treatment of soybean lectin on the HeLa cells shows the dose dependent cell death of cancer cells. The viability of cancer cells are decreasing with increase of the drug concentration. Further studies can be done on induction of any cell death like apoptosis, autophagy and necrosis by Sbl and the pathway involving in the cell death process.

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